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Selective Inhibition of Functional Lymphocyte Subpopulations by Ribavirin

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The present studies were designed to examine the effects of ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a broad-spectrum antiviral agent, on the generation of murine antibody responses in vitro. Whereas primary and secondary sheep erythrocyte-specific, plaque-forming cell responses by normal murine spleen cells were enhanced by low concentrations of ribavirin (1 μg per culture), they were strongly inhibited by higher concentrations of ribavirin (5 to 10 μg per culture). Both phenomena occurred with the greatest magnitude when spleen cells were exposed to ribavirin 48 to 72 h after culture initiation. Enhancement appeared to result from selective interference with suppressor T cells, since ribavirin failed to augment lipopolysaccharide-specific plaque-forming cell responses in T cell-depleted spleen cell cultures but inhibited concanavalin A-induced lymphocyte proliferation and suppressor T cell generation in cultures of normal spleen cells. The immunosuppressive properties of ribavirin were mediated by a direct antiproliferative effect and, at higher concentrations, a cytotoxic effect for B lymphocytes, since the drug inhibited plaque-forming cell responses in T cell-depleted spleen cell cultures, suppressed lipopolysaccharide-induced lymphocyte proliferation, and reduced viable spleen cell recoveries.

Ribavirin, a synthetic inhibitor of RNA and DNA virus replication, has displayed considerable chemotherapeutic activity against experimentally induced viral infections and is currently undergoing extensive clinical evaluations in humans (24, 26, 27). Some of its most promising features are a broad spectrum of activity (the broadest of the currently studied antiviral agents), a relative lack of toxicity for normal or virus-infected host cells, and the failure of viral strains exposed to ribavirin to develop drug resistance. Although ribavirin inhibits replication of some viruses by specifically interfering with RNA polymerases or by preventing the 5'-terminal modification of viral mRNA, several observations suggest the possibility that some of the effectiveness of the drug in vivo may stem from its immunosuppressive activity. For example, ribavirin is most beneficial against influenza and vaccinia virus infections, diseases in which hypersensitivity responses to viral antigens may contribute significantly to pathogenicity (2, 23, 30). Ribavirin is also active in the treatment of murine systemic lupus erythematosus (10, 11). More recently, we have shown that administration of ribavirin to mice in a single therapeutic dose (40 mg/kg) inhibits primary immunoglobulin M (IgM) and IgG plaque-forming cell (PFC) responses and suppresses memory cell generation without significant loss of lymphoid cells (19).

In an attempt to identify the mechanism of immunosuppression, the capacity of ribavirin to alter antigen-dependent PFC responses in cultures of normal and T cell-depleted murine spleen cell populations was examined. The results obtained indicate that ribavirin, at concentrations that are antiviral in vitro, exerted a differential effect on lymphocyte subpopulations participating in antibody responses. Low concentrations (0.1 to 1 μg/ml) of ribavirin moderately enhanced PFC responses, whereas slightly higher concentrations (5 to 10 μg/ml) abolished both primary and secondary PFC responses. Enhancement resulted from selective inhibition of suppressor T cells, whereas the primary targets for the immunosuppressive effects of ribavirin were B cells.

MATERIALS AND METHODS

Mice. C57BL/6 mice of both sexes were purchased from Jackson Laboratories, Bar Harbor, Maine, and housed in cages with barrier filters.

Ribavirin. 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin, Viratek, Inc., Covina, Calif.) was stored at room temperature as a dry powder. Ribavirin was dissolved in Hanks balanced salt solution (HBSS; Microbiological Associates, Bethesda, Md.) and sterilized by filtration (0.45-μm-pore filters; Millipore Corp., Bedford, Mass.) immediately before use.

Antigens. Sheep erythrocytes (SRBC) in Alsever solution (Randolph Biologicals, Houston, Tex.) were washed three times in HBSS. Packed SRBC were

suspended in HBSS at a final concentration of 5 or 10% (vol/vol). Bacterial lipopolysaccharide (LPS), extracted from *Escherichia coli* O:127:B8 by the Westphal technique, was purchased from Difco Laboratories, Detroit, Mich., stored at 4°C, and suspended in HBSS.

Con A. Chromatographically purified concanavalin A (ConA; Pharmacia Fine Chemicals, Uppsala, Sweden) was stored at −20°C. ConA solution was freshly prepared in HBSS on the day of its use.

Cell culture system. A completely supplemented Eagle minimal essential medium containing 10% fetal calf serum (lot no. 97310; Microbiological Associates), 10 U of gentamycin per ml, and 5×10^{-5} M 2-mercaptoethanol (Eastman Organic Chemicals, Rochester, N.Y.) was used in all experiments. Techniques for preparation of spleen cell suspensions and for generation of in vitro PFC responses to SRBC and LPS have been described previously (14, 15). Immunoglobulin class-specific PFC were enumerated 5 days after culture initiation by the Jerne plaque technique. This assay provides a method of assessing B cell function by measuring antibody (plaque)-forming cells after stimulation with antigen. Results are expressed as the mean PFC response per 10^6 cells recovered from duplicate cultures, or as the percentage of the control response of untreated cultures. For statistical comparison, results from three to five experiments with identical protocols were pooled by normalization of data.

Suppressor T cell activation. Details of the capacity of ConA-activated spleen cells to inhibit PFC responses in vitro have been published elsewhere (3, 9, 21). Briefly, 10^7 spleen cells in 1 ml of tissue culture medium were incubated for 48 h with and without 1 μg of ConA. Cells were harvested, washed thoroughly, and counted, and 10^5 cells were added to newly initiated spleen cell cultures stimulated with SRBC.

Depletion of spleen cell populations of thymus-dependent lymphocytes. Depletion of T cells from spleen cell cultures was accomplished by incubating the cultures with a monoclonal antibody directed against the theta (θ) or Thy 1 surface marker present on thymus-derived lymphocytes. Briefly, C57BL/6 spleen cells (10^7 cells per ml in HBSS plus 5% fetal calf serum) were incubated at 4°C for 30 min with a 1:1,000 final dilution of monoclonal anti-Thy 1.2 IgM antibody (New England Nuclear Corp., Boston, Mass.). Guinea pig complement (University of Texas System Cancer Center, Bastrop) was then added at a 1:5 dilution, and the mixture was incubated at 37°C for 45 min. The remaining cells were washed three times in HBSS plus 5% fetal calf serum, suspended in fresh tissue culture medium, and added immediately to culture dishes.

Mitogen-induced lymphocyte proliferation. Since ribavirin markedly increases intracellular dTTP pools (18) but not ATP or dATP pools, incorporation of [^{3}H]adenosine rather than [^{3}H]thymidine was used to measure lymphocyte proliferation in ribavirin-treated cultures (17, 18). Spleen cells 5×10^5 in 0.1 ml of tissue culture medium were added to each well of a flat-bottomed culture plate (Microtest II; Falcon Plastics, Oxnard, Calif.)

ConA (1 μg), LPS (5 μg), or ribavirin (0 to 2 μg) was added individually in 0.05 ml of HBSS so that the final volume of each well was 0.20 ml. Plates were incubated at 37°C for 48 h. [^{3}H]adenosine (0.5 Ci; 16.3 Ci/mol; New England Nuclear Corp.) was added for the final 4 h of incubation. At termination of incubation, cells were harvested and prepared for liquid scintillation counting as described previously (18). Data from three replicate cultures were used to calculate the mean counts per minute per culture ± the standard error of the mean. Inhibition of proliferation by ribavirin was also expressed as the percentage of control incorporation.

Cell viability. Cells from duplicate cultures were harvested and pooled in glass tubes (12 by 75 mm). Recovered cells were counted with a Coulter electronic cell counter, and percent viability was determined by trypan blue exclusion.

Statistics. Comparisons between PFC and proliferative responses in control and ribavirin-treated cultures and differences in viable cell recoveries were analyzed by Student's *t* test.

RESULTS

Modulation of primary and secondary PFC responses by ribavirin. Figure 1 illustrates the results of five experiments in which various amounts of ribavirin were added to spleen cell cultures stimulated with T cell-dependent

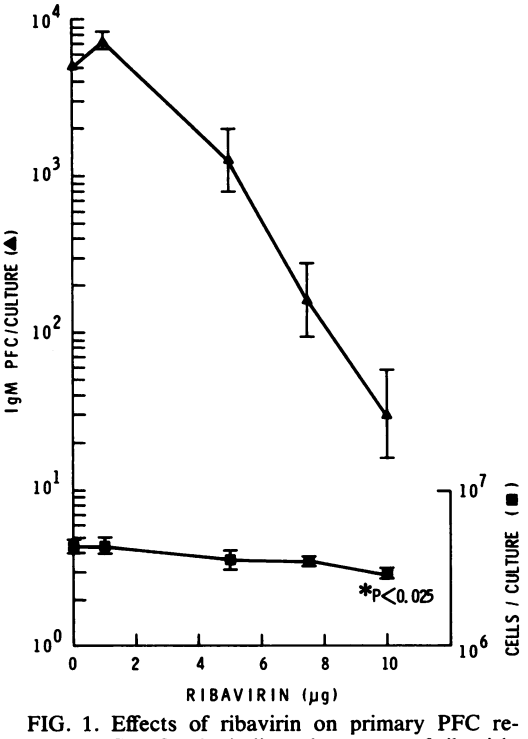

FIG. 1. Effects of ribavirin on primary PFC responses to SRBC. The indicated amounts of ribavirin were added with SRBC at the initiation of C57BL/6 spleen cell cultures. SRBC-specific PFC responses and nucleated cell recoveries were measured on day 5. Data from identical experiments were normalized to a control response of 5,000 PFC per culture and 4.35×10^6 cells per culture; each point represents the mean ± standard error of the mean, determined by Student's *t* test.

(SRBC) or T cell-independent (LPS) antigens. A significant ($P < 0.01$) increase in the primary PFC response to SRBC was observed with 1 μg of ribavirin; however, this response decreased progressively with 5, 7.5, and 10 μg of ribavirin (Fig. 1). No significant reduction in spleen cell recoveries occurred, except in cultures receiving 10 μg of ribavirin. Ribavirin also exerted similar dose-dependent effects on the primary PFC response to LPS (Fig. 2). Enhancement, seen in Fig. 2 with 0.1- and 1-μg doses, was followed at higher doses by a marked reduction in the PFC response. There was a loss of viable cells at the highest dose tested. By using spleen cells from SRBC-primed mice, secondary IgM and IgG PFC responses were enhanced and suppressed to the same degree by the same doses of ribavirin that modulated the primary PFC responses (data not shown).

Effects of length of exposure to ribavirin on PFC responses. To identify the interval required for ribavirin to exert maximal effects, SRBC-stimulated spleen cells were exposed to 1 or 10 μg of ribavirin at culture initiation or at various times thereafter. Each exposure was terminated after 24 h by thoroughly washing the spleen cell suspension and culture dishes with fresh tissue culture medium. Spleen cells were then returned to their original dishes and incubated for the remainder of the 5-day culture period. When 1 μg of ribavirin was administered in successive 24-h periods, IgM PFC responses progressively increased to nearly double those in untreated control cultures in the period from 48 to 72 h (Fig. 3). A 1-μg dose of ribavirin given from 48 to 72 h was even more successful in enhancing PFC responses than a continuous pulse from 0 to 120 h; however, delaying the addition of ribavirin until 72 or 96 h after culture initiation had diminished effects. Results from identical experiments, except for the use of 10-μg doses, resulted in a progressive increase in the suppression of PFC responses that was maximal at 48 to 72 h.

These experiments indicated that the maximal effects of ribavirin occurred 48 to 72 h after culture initiation. Therefore, this time period was selected to assess the lowest exposure of ribavirin necessary to produce the greatest suppressive effects. In addition, because of the ability of guanosine to reverse many of the effects of ribavirin, the same protocol was used to evaluate guanosine interference with ribavirin action. A 10-μg dose of ribavirin was added 60 h

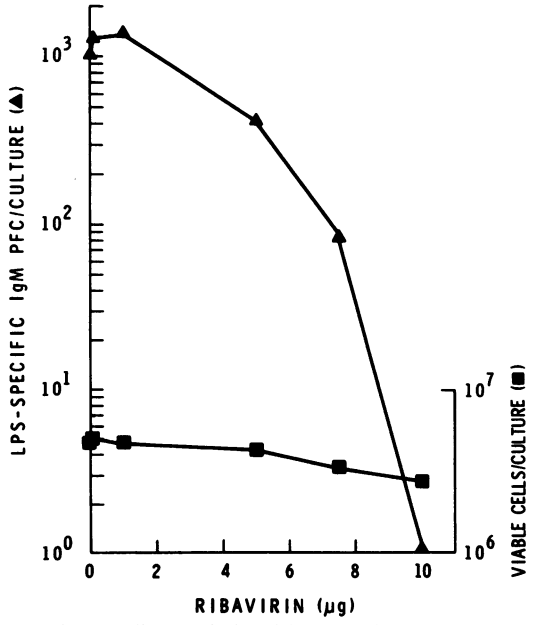

FIG. 2. Effects of ribavirin on primary PFC responses to LPS. *E. coli* LPS and ribavirin were added simultaneously at the initiation of C57BL/6 spleen cell cultures. LPS-specific PFC responses and viable cell recoveries were determined on day 5. Results are from a representative experiment.

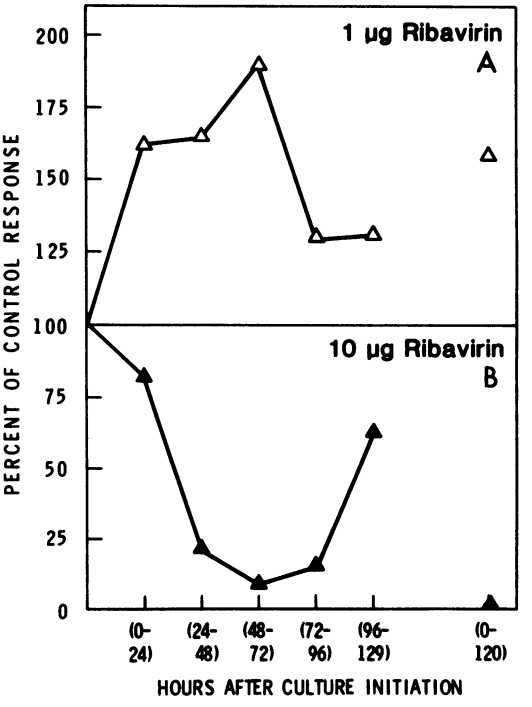

FIG. 3. Determination of time of maximal effect of ribavirin on lymphocytes in culture. SRBC-stimulated C57BL/6 spleen cells were pulsed with 1 (A) or 10 (B) μg of ribavirin at each of five consecutive 24-h periods. Periods of drug exposure were terminated by harvesting and washing spleen cell cultures as described in the text. The percentage of control response was calculated from cultures not exposed to ribavirin but otherwise treated identically.

(the midpoint) after initiation of SRBC-stimulated spleen cell cultures. After various lengths of time, each exposure period was terminated as described above, and spleen cells were returned to their dishes for the duration of the 5-day incubation. Other cultures were exposed to 10 μg each of ribavirin and guanosine per ml for the same time periods. When ribavirin or ribavirin and guanosine were either added and removed immediately or left in the cultures for 6 h, IgM PFC responses were not significantly affected (Fig. 4). However, pulses of 12, 18, 24, and 36 h led to a progressive reduction of the PFC response. At 12 h, 10 μg of guanosine entirely prevented the inhibitory effects of ribavirin; guanosine, however, did not interfere with ribavirin when both compounds were present together for 18, 24, or 36 h, presumably due to irreversible effects of ribavirin on the cells.

Specificity of nucleosides inhibiting the immunosuppressive effects of ribavirin. To determine if ribavirin suppresses PFC responses in vitro by blocking a specific step in cellular metabolism, the capacity of other soluble nucleosides to inhibit ribavirin was also examined. Table 1 illustrates that when administered as a 12-h pulse 48 h after culture initiation, 10 μg of ribavirin inhibited IgM PFC responses to 21% of the control response. The simultaneous addition of 10 μg of guanosine, but not adenosine, inosine, or xanthosine, with 10 μg of ribavirin completely restored the PFC response. Table 1 also shows that, except for inosine, the addition of these nucleosides individually to SRBC-stimulated spleen cell cultures caused slight or no suppression of the PFC response.

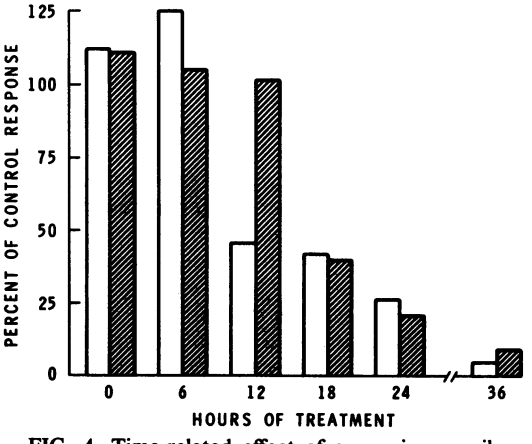

FIG. 4. Time-related effect of guanosine on ribavirin interference with the PFC response. Either 10 μg of ribavirin (open bars) or 10 μg of ribavirin and 10 μg of guanosine (cross-hatched bars) were added to spleen cell cultures at 48 h after stimulation with SRBC. Ribavirin and guanosine were removed after the indicated period of exposure as described in the text. The percentage of control response was calculated from SRBC-stimulated cultures that were not exposed to ribavirin or guanosine but were washed and replated in parallel with other cultures.

TABLE 1. Inhibition of the immunosuppressive activity of ribavirin[a]

Nucleoside	% Inhibition[b] of effects of ribavirin at indicated dose (μg)	
	0	10
None	100[b]	21[b]
Adenosine	104	41
Inosine	72	50
Guanosine	115	114
Xanthosine	90	41

[a] At 48 h after stimulation with SRBC, spleen cell cultures were treated either with 10 μg of ribavirin and 10 μg of the indicated nucleoside or with 10 μg of nucleoside alone. After 12 h of exposure, cells were washed thoroughly and returned to cultures for the remainder of the 5-day incubation period.

[b] Percentage of control response, based on a control level of 437 IgM PFC per 10^6 cells.

Effects of ribavirin on PFC responses of T cell-depleted spleen cell populations. The capacity of ribavirin to exert both positive and negative influences on primary and secondary antibody responses suggests a selective interference with different lymphocyte subpopulations. T cell-depleted spleen cell cultures were used to assess the direct effects of ribavirin on B cell responses (Table 2). These responses were obtained by measuring LPS-specific PFC responses, uninfluenced by T cells. After T cells were depleted by monoclonal anti-Thy 1.2 IgM antibody and complement, C57BL/6 spleen cells generated an adjusted mean response of 200 PFC per 10^6 cells. The addition of 0.1 or 1 μg of ribavirin failed to enhance PFC responses; in fact, 1 μg suppressed PFC responses significantly (56%; $P = 0.05$). The addition of 10 μg of ribavirin inhibited PFC responses to less than 1% of the control responses. In parallel cultures (Table 2), 1 μg of ribavirin enhanced LPS- and SRBC-specific PFC responses of untreated C57BL/6 spleen cells.

Ribavirin interference with suppressor T cell generation. Murine splenic PFC responses are suppressed by the addition of mitogenic concentrations of ConA at culture initiation but are enhanced if the addition of ConA is delayed until 48 h thereafter. These effects result from nonspecific activation of a large number of suppressor T cells and helper T cells, respectively (4, 28). Initial experiments demonstrated that exposure of spleen cells to 1 μg of ConA or 1 μg of ribavirin 48 to 72 h after culture initiation moderately enhanced IgM PFC responses, whereas the simultaneous addition of 1 μg of ribavirin and 1

TABLE 2. Failure of ribavirin to enhance PFC responses in T cell-depleted spleen cell cultures

Spleen cell cultures[a]		Ribavirin dose (μg)	IgM PFC per 10^6 cells[b] in expt			Mean IgM PFC per 10^6 cells[c] ± SEM	P[d]	% of control response
Treatment	Antigen		1	2	3			
None		0	124	308	380	200		100
	LPS	0.1	228	389	448	304 ± 34	0.025	152
		1	221	423	612	318 ± 24	0.01	159
		10	14	0	5	9 ± 7	0.0025	5
	SRBC	0	998	1,094	1,344	1,000		100
		1	1,498	1,491	1,785	1,397 ± 53	0.005	140
T cell depleted	LPS	0	258	313	903	200		100
		0.1	175	295	853	171 ± 18	0.1	86
		1	33	294	539	111 ± 49	0.05	56
		10	3	0	3	1 ± 1	0.0005	1
	SRBC	0	333	10	131	100		100
		1	261	28	62	135 ± 73	0.25	135

[a] C57BL/6 spleen cells were either treated with monoclonal anti-Thy 1.2 serum and complement (T cell depleted) or not treated (none). A total of 10^7 cells from each group were incubated with LPS or 5×10^7 SRBC and the indicated concentration of ribavirin.

[b] Assayed on day 5.

[c] Responses of untreated spleen cells were normalized to 200 LPS-specific and 1000 SRBC-specific PFC per 10^6 recovered cells, and responses of T cell-depleted spleen cells were normalized to 200 LPS-specific and 100 SRBC-specific PFC per 10^6 recovered cells.

[d] Determined by Student's t test.

μg of ConA resulted in an increase in the PFC response greater than that observed with ConA or ribavirin alone. In contrast, low doses (1 to 2 μg) of ribavirin appeared to counteract the suppressive effects of ConA when spleen cells were treated at culture initiation (data not shown).

These results suggested that ribavirin did not interfere with the activation of helper T cells but inhibited the generation of suppressor T cells. More direct evidence for this hypothesis was obtained in the following experiment. Normal spleen cells were incubated for 24 h with or without ConA and various amounts of ribavirin. Cells were recovered, washed, counted, and transferred to newly initiated spleen cell cultures stimulated with SRBC. Data presented in Fig. 5 demonstrate that PFC responses in cultures receiving 10^5 ConA-activated spleen cells were markedly lower than in cultures receiving the same number of unstimulated spleen cells and that spleen cells activated in the presence of ConA and 1 or 10 μg of ribavirin possessed progressively less inhibitory activity.

The capacity of ribavirin to selectively interfere with the proliferative responses of lymphocyte subpopulations was examined by using spleen cell cultures stimulated with mitogenic concentrations of ConA or LPS. The data in Table 3 indicate that ribavirin suppressed not only the proliferative response of T cells to ConA stimulation but also suppressed the B cell response to LPS stimulation. It is particularly noteworthy, however, that 0.5 and 1 μg of ribavirin, amounts which enhanced PFC responses, inhibited proliferation induced by ConA without altering the response to LPS. Statistical analysis showed that these results were significant at the $P < 0.001$ level, indicating a greater sensitivity of ConA-activated spleen cells to ribavirin.

DISCUSSION

Although ribavirin has been used successfully for the treatment of naturally acquired and experimentally induced viral infections of humans and laboratory animals, only recently has it been found to possess immunoregulatory activity. When given to mice after antigen administration, ribavirin suppresses primary PFC responses and memory cell generation without apparent lymphocytotoxicity (19). Other studies from this laboratory have shown that ribavirin stimulates serum hemagglutination inhibition antibody responses when administered as an aerosol to human volunteers acutely ill with influenza A virus infections (12). By using an in vitro system, the present investigation was designed to examine the capacity of ribavirin to modulate antibody responses, to identify its cellular targets, and to determine its mechanism(s) of action. The results show that, depending on the dose and duration of drug exposure, ribavirin may moderately enhance or entirely inhibit the generation of PFC responses. A ribavirin dose of 5 to 10 μg per culture was strikingly immunosuppressive, since this dose inhibited both IgM and

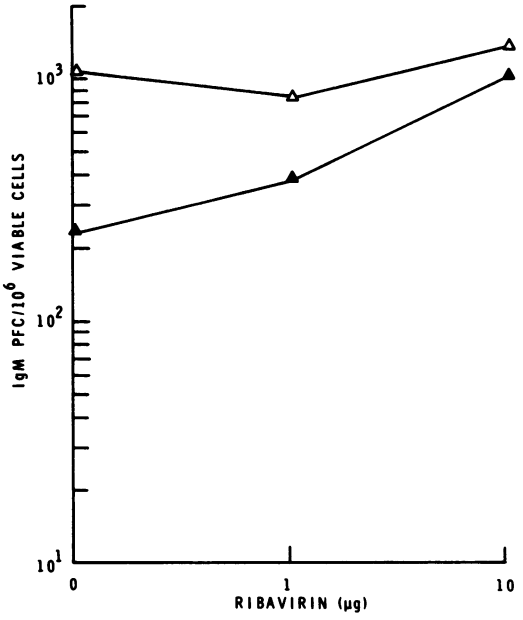

FIG. 5. Interference with suppressor T cell generation by ribavirin. We incubated 10^7 spleen cells with (▲) or without (△) 1 μg of ConA and the indicated amounts of ribavirin. After 24 h, cells were washed, counted, and added to newly established spleen cell cultures stimulated with SRBC. PFC responses were measured on day 5.

IgG plaque cell formation in primary and secondary responses.

The modulation of PFC responses to both T cell-dependent (SRBC) and T cell-independent (LPS) antigens over an extremely narrow range of ribavirin concentrations suggests that these effects are mediated by a similar mechanism acting on at least two distinct cell types. It is unlikely that ribavirin interferes with the function of macrophages, since the drug exerted its maximal effects when administered 48 to 96 h after culture initiation, a period during which macrophages are not required for the development of optimal PFC responses (5, 20). Our results demonstrate that immunosuppressive activity results from a direct effect on B cells, since ribavirin inhibited LPS-specific PFC responses in T cell-depleted spleen cell cultures and decreased proliferation of mitogen-stimulated B lymphoblasts. In contrast, enhancement appears to be due to selective elimination of suppressor T cells, since low doses of ribavirin failed to increase PFC responses in the absence of mature T cells (Table 2), inhibited T cell proliferation but not B cell proliferation (Table 3), and blocked the generation of mitogen-induced suppressor T cells (Fig. 5). It is not possible to conclude from the data presented what the effect of ribavirin on helper T cells is. However, ribavirin did not interfere with the capacity of soluble ConA to augment the PFC response that is mediated by helper T cell activation (28).

TABLE 3. Suppression of T cell and B cell proliferation by ribavirin[a]

Mitogen	Ribavirin dose (μg)	[^{3}H]adenosine incorporation (cpm ± SEM)	% of control response
ConA (T cell)	0	36,189 ± 3,091	100
	0.5	29,458 ± 2,572	82
	1	23,316 ± 1,040	66
	2.5	12,405 ± 487	35
	5	7,596 ± 1,213	22
	10	4,014 ± 434	11
LPS (B cell)	0	45,898 ± 2,891	100
	0.5	46,715 ± 2,823	104
	1	40,587 ± 2,635	90
	2.5	23,352 ± 2,322	51
	5	11,165 ± 1,372	24
	10	7,182 ± 756	16

[a] Statistical analysis: ConA versus LPS, $P < 0.001$ (t, 3.18; degrees of freedom, 30).

This investigation further reveals the cytotoxicity of ribavirin in moderate doses for lymphocytes in vitro. Ribavirin appears to have a broad spectrum of dose-related effects. At low doses it interfered with suppressor T cell function. At higher doses, all lymphocyte populations were suppressed, and with still higher doses, irreversible toxicity occurred. Previous reports investigating the antiviral activity of ribavirin have not described toxicity for several cell lines used to support virus replication (8, 16, 26). Lowe et al. monitored the metabolic effects of ribavirin on RK-13 cells and tumor cell lines and determined that although ribavirin inhibited the rate of growth, decreases in cell viability only occurred when doses of 500 to 1,000 μg/ml were used (13).

The suppression of PFC responses by ribavirin was almost always accompanied by a significant loss of viable spleen cells. Reductions in viable cell recoveries were conspicuous when 5 μg of ribavirin was added to spleen cell cultures for 5 days or when 10 μg of ribavirin was added for as short a period as 24 h. In addition, the inability of guanosine to inhibit suppression by 10 μg of ribavirin after 12 h suggests that cytotoxicity contributes in an important way to immunosuppressive activity. Just why lymphocytes are more susceptible to ribavirin than other cell types may be because of several unique characteristics of their purine metabolism. Inhibition of inosine monophosphate dehydrogenase by ribavirin in all cells leads to a rapid loss of guanosine nucleotides; however, the limited capacity of lymphocytes to synthesize GMP from the de novo purine pathway may result in prolonged GMP deprivation (13, 22, 25). Moreover, inhibition of inosine monophosphate syn-

thesis by ribavirin would also be expected to cause accumulation of inosine, hypoxanthine, and other intermediates of the purine interconversion pathway. It is of considerable interest that ribavirin-treated human lymphoblasts secrete inosine and hypoxanthine extracellularly (29) and that the addition of inosine to murine spleen cell suspensions inhibited PFC responses (Table 1). In addition, intracellular accumulation of deoxyinosine, deoxyguanosine, and deoxyadenosine has recently been shown to be lymphocytotoxic in vitro and is thought to be responsible for the severe immunological dysfunction observed in T and B lymphocytes of patients with adenosine deaminase and purine nucleoside phosphorylase deficiencies (1, 6, 7).

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